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sem-4 Promotes Vulval Cell-Fate Determination in *Caenorhabditis elegans* through Regulation of *lin-39* Hox

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Vulval cell-fate determination in *Caenorhabditis elegans* requires the action of numerous gene products, including components of the Ras/Raf/MAPK signaling cascade and the hox gene *lin-39*. *sem-4* encodes a zinc finger protein with previously characterized roles in fate specification of sex myoblasts, coelomocytes, and multiple neuronal lineages in *C. elegans* (M. Basson and R. Horvitz, 1996, *Genes Dev.* 10, 1953–1965). By characterizing three new alleles of *sem-4* that we identified in a screen for vulval-defective mutants, we determined that loss of *sem-4* activity results in abnormal specification of the secondary vulval cell lineages. We analyzed *sem-4* interactions with other genes involved in vulval differentiation and determined that *sem-4* does not function directly in the Ras-mediated signal transduction pathway but acts in close association with and upstream of *lin-39* to promote vulval cell fate. We demonstrate that *sem-4* regulates *lin-39* expression and propose that *sem-4* is a regulator of *lin-39* in the vulval cell-fate determination pathway that may act to link *lin-39* to incoming signals.

***Key Words:* *Caenorhabditis elegans*; *lin-39*; Ras signaling; *sem-4*; vulval development.**

INTRODUCTION

It is becoming increasingly evident that simplistic models of cell-fate induction are inadequate. Fate determination often relies on the intricate convergence of multiple signaling pathways. Vulval development in *Caenorhabditis elegans* provides an excellent model system to study mechanisms by which various pathways converge to specify cell fate. Critical events in vulval development occur during the four larval stages (L1–L4) of *C. elegans* development (Sternberg and Horvitz, 1986; Sulston and Horvitz, 1977). During L1, 12 P*n*.p cells are born on the ventral surface of the hermaphrodite. P1.p, P2.p, P9.p, P10.p, and P11.p fuse with a syncytial hypodermal cell (F fate) in L1 (Fig. 1). P3.p adopts an F fate during L2 in approximately 50% of animals. In the remaining animals, P3.p becomes part of the vulval equivalence group with P4–8.p (Fig. 1). The 6 unfused vulval precursor cells (VPCs) have the potential to adopt vulval fates during L3. However, in a wild-type animal only P5.p, P6.p, and P7.p execute vulval cell fates and undergo vulval morphogenesis during L3 and L4. The remaining 2 or 3 VPCs adopt a nonvulval, 3° fate, dividing once and fusing to the syncytial, hypodermal cell hyp7 (Fig. 1).

Specification of vulval cell fate requires inductive signaling mediated by a conserved Ras/Raf/MAPK pathway and lateral signaling mediated by the conserved *lin-12* Notch protein (reviewed in Greenwald, 1997; Kornfield, 1997; Sternberg and Han, 1998). Appropriate signal transduction causes P6.p to adopt a 1° vulval cell fate and P5.p and P7.p to adopt 2° vulval cell fates (Fig. 1).

While the functions of the primary components of the Ras pathway have been well elucidated, little is known about the genes that act downstream of Ras-mediated signaling in executing vulval cell fates. The homeotic gene, *lin-39*, a homolog of sex-combs-reduced, plays a direct role in vulval cell induction during L3 (Clandinin *et al.*, 1997; Maloof and Kenyon, 1998). Additionally, *lin-39* activity is required during L1 to prevent P4.p–P8.p from fusing with hyp7, allowing these cells to remain competent to respond to Ras signaling during L3 (Clark *et al.*, 1993; Wang *et al.*, 1993). Loss of *lin-39* activity prior to L3 results in inappropriate adoption of an F fate by P4–8.p. Loss of *lin-39* activity in L3 results in adoption of nonvulval 3° fates. Up-

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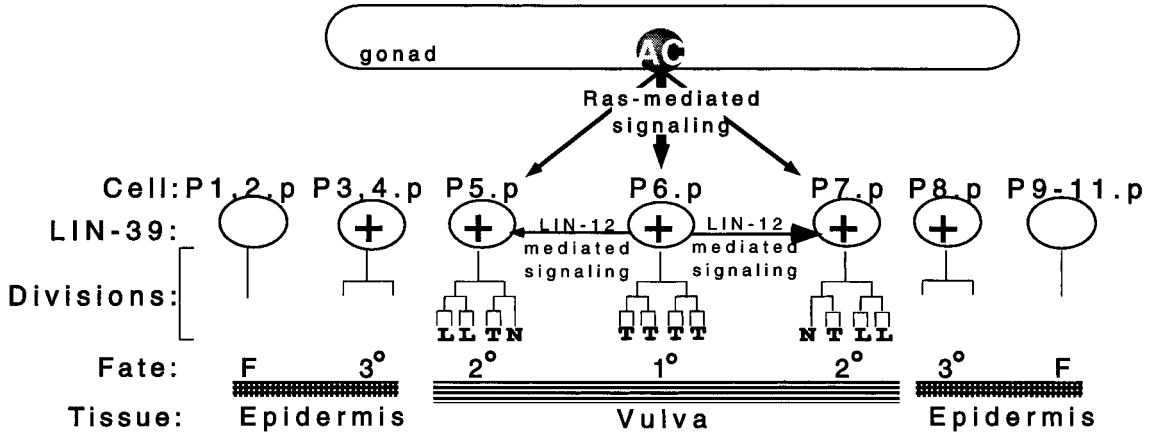

FIG. 1. Overview of vulval development. 12 P*n*.p cells are born on the ventral surface of the hermaphrodite during L1. P3.p–P8.p express *lin-39* and become the vulval precursor cells (VPCs), whereas the outer P*n*.p cells do not express *lin-39* and consequently adopt fused (F) fates. (P12.p adopts a unique fate not described here.) A signal from the anchor cell (AC) in the somatic gonad induces the closest VPCs to adopt vulval fates. The remaining VPCs adopt nonvulval 3° fates. Division patterns for each fate are indicated. L, T, and N represent lateral, transverse, and no division, respectively, for the final round of division. Arrows indicate signaling events between cells utilizing the indicated signaling pathways.

regulation of *lin-39* expression occurs during normal vulval induction and is dependent on an intact Ras signaling cascade, indicating that *lin-39* expression is regulated by Ras-mediated signaling (Clandinin *et al.*, 1997; Maloof and Kenyon, 1998).

Wingless (wnt)-mediated signaling has also been implicated in vulval development (Eisenmann *et al.*, 1998; Hoier *et al.*, 2000; Sawa *et al.*, 1996; Sternberg and Horvitz, 1988; Thorpe *et al.*, 1997). Regulation of *lin-39* was first implicated in this wnt signaling event based on the characterization of a mutation in the *bar-1* gene, a *C. elegans* homolog of β-catenin (Eisenmann *et al.*, 1998). β-Catenins can act as downstream mediators of wnt signaling (reviewed in Dierick and Bejsovec, 1999). Loss of *bar-1* activity is associated with a decrease in *lin-39* expression in the VPCs, and inappropriate vulval cell fate determination that can be partially rescued by ectopic expression of *lin-39*, suggesting that *bar-1* acts to regulate or maintain expression of *lin-39* in the vulval cells during induction (Eisenmann *et al.*, 1998).

We show here that *sem-4* also plays a role in the regulation of *lin-39*. The *sem-4* gene was defined by mutations that caused a sex myoblast defect. *sem-4* encodes a putative transcription factor with seven C_2H_2-type zinc fingers (Basson and Horvitz, 1996) with homology to the zinc fingers of the *Drosophila* homeotic gene *spalt* (Jürgens, 1988; Kühnlein *et al.*, 1994) and the human transcription factor PRDII-BF1 (Seeler *et al.*, 1994). Basson and Horvitz (1996) characterized three types of *sem-4* alleles: null alleles, an allele associated primarily with mesodermal lineage defects (*n1378*), and an allele associated primarily with neuronal lineage defects (*n2654*). In the null mutants, the mesodermal-derived sex myoblasts fail to differentiate, resulting in an egg-laying-defective (Egl) phenotype, and fate determination of many neuronal cells is abnormal, contributing to a defecation defect (Basson and Horvitz, 1996). *n1378* mutants are Egl but have few aberrant neuronal lineages while *n2654* mutants have defecation defects but are non-Egl. The phenotypes associated with *n1378* and *n2654* are similar in severity to those associated with a null allele, suggesting that, although not null, *n1378* and *n2654* are strong loss-of-function mutations in the sex-myoblast and neuronal lineages, respectively, while retaining activity in other tissues.

We identified three additional *sem-4* alleles based on a vulval lineage-defective phenotype and have demonstrated that a null allele and the mesodermal-affected allele also cause this previously uncharacterized phenotype. We present genetic evidence that *sem-4* acts in close association with *lin-39* to promote vulval cell fate and that *sem-4* regulates *lin-39* expression. We propose that *sem-4* is an upstream regulator of *lin-39* in the vulval cell fate determination pathway and may act to link *lin-39* to other cell-type-specific cues.

MATERIALS AND METHODS

General Methods and Strains

Strains were maintained at 20°C, unless otherwise noted, using standard methods (Brenner, 1974; Wood, 1988). Zeiss Axioskop and fluorescence-equipped Axioplan-2 microscopes were used to analyze phenotypes. The following alleles were utilized (Riddle *et al.*, 1997, or as referenced): N2 (Bristol), *muIs23[hs::lin-39 dpy-20(+)]* (Maloof and Kenyon, 1998), *kuIs35[sem-4::gfp unc-119(+)]* and *kuEx113[sem-4::gfp unc-119(+)]* (this work), LG I—*dpy-5(e61)*,

let-386(h117), sem-4(n1971, n1378, n2654) (Basson and Horvitz, 1996), *ku200, ku206, ku210* (this work), *unc-13(e450),* LG III—*lin-39(n709ts), mab-5(e1239), unc-36(e251), dpy-19(e1259), unc-32(e189), lin-12(n137n720 lf), lin-12(n137 gf), unc-119(ed3),* LG IV—*lin-1(e1777), lin-45(ku112)* (Sundaram and Han, 1995), *let-60(n1046), dpy-20(e1282), muIs6[lin-39::lacZ]* (Wang *et al.,* 1993), *kuIs34[sem-4::gfp unc-119(+)]* (this work), LG V—*him-5(e1490).*

Isolation of sem-4 and Characterization of Alleles

sem-4 alleles were isolated in a screen for vulval defective mutants, as previously described (Hanna-Rose and Han, 1999). *ku200, ku206,* and *ku210* were outcrossed six times and failed to complement (data not shown). Genetic mapping placed *ku200* close to *sem-4* (e.g., 22/22 Dpy-5 non-Let-386 segregated Egl and 21/49 Unc-13 non-Let-386 segregated Egl). The cosmid F15C11, which encodes the *sem-4* gene, rescued the *ku200* mutant phenotype, and *ku200* failed to complement *sem-4(n1378* and *n1971)* (data not shown). Additionally, *ku200* mutants lacked sex myoblasts (0/30 sex myoblasts present) and coelomocytes (9/19 coelomocytes present), known *sem-4* mutant defects.

Analysis of sem-4::gfp Expression

The *sem-4::gfp* construct pMK212 (gift from M. Koelle, Yale School of Medicine) is a translational fusion containing a *Sph*I–*Pst*I genomic fragment, encoding approximately half of SEM-4, fused in frame to GFP. *kuEx113* was formed by injecting pMK212 and an *unc-119*-rescuing construct (Maduro and Pilgrim, 1995) into *unc-119(ed3)* hermaphrodites at a concentration of 50 ng/μl each. Integration of *kuEx113* was induced by γ-irradiation to form *kuIs34* and *kuIs35.*

Construction of Double Mutants

Doubles with *mab-5(e1239)* and *lin-39(n709ts)* were made by crossing *ku200*/+; *unc-36(e251)*/+ males to each mutant and selecting Egl non-Unc F_2 hermaphrodites that segregated Unc progeny and Egl non-Unc F_3 hermaphrodites that no longer segregated Unc. The presence of *e1239* was verified by sequencing. To create *sem-4; let-60, let-60 ras(n1046 gf)* was crossed to *ku200*/+ males and Egl Muv F_3 progeny were selected from F_2 Egl animals. *lin-45(ku112); dpy-20(e1282)* was crossed to *ku200*/+ males and Dpy Egl F_2 progeny were selected to create the *sem-4; lin-45* double.

Doubles between *kuIs34[sem-4::gfp]* and *lin-12* alleles were created by crossing *kuIs34*/+ males to *unc-32(e189) lin-12(n137n720lf)/dpy-19(e1259) lin-12(n137gf)* hermaphrodites. F1 progeny that segregated fluorescent animals and either Dpy or Unc animals were selected. F2 progeny that were homozygous for *kuIs34* and heterozygous for each of the marked *lin-12* alleles were selected and maintained as strains.

β-Galactosidase and LIN-39 Detection

Two methods were used to observe β-galactosidase staining in *muIs6*-containing animals. Animals were washed in M9, pelleted, washed in 4°C acetone, and dried in the Spin-Vac. Staining was performed overnight at room temperature in 0.5 M NaPi monobasic, 0.5 M NaPi dibasic, 1 M $MgCl_2$, 100 mM K-ferrocynate, 100 mM K-ferricynate, 1% SDS, 2% X-Gal + 50 μg/ml DAPI. To better preserve morphology, animals were washed in M9, pelleted, and stained as above. We performed immunofluorescence using anti-β-gal primary antibody (Promega) and donkey anti-mouse secondary antibody conjugated to Cy-3 (Jackson Laboratories) and using anti-LIN-39 antibodies as previously described (Bettinger *et al.,* 1996; Maloof and Kenyon, 1998).

Heat-Shock Protocol

A manual heat-pulsing method, intended to mimic the protocol of Maloof and Kenyon (1998), was used. Eggs, obtained by dissecting gravid adults, were allowed to hatch for 3 h. The resulting synchronized L1 hermaphrodites were maintained at 20°C for 17 h. Prior to L3, they were shifted to 30°C for 2 h and allowed to recover for 2 h at 20°C. This 4-h cycle was repeated until the animals reached L4. The number of temperature shifts varied because the heat shock construct in combination with the heat pulses slows growth. This heat-pulsing continued for up to 30 h.

Construction of col-10::lin-39

The entire *lin-39* cDNA was amplified from pCH17.1 (Wang *et al.,* 1993), using a 5′ primer with an *Nhe*I cloning site and the ATG start codon, GCTAGCGCTAGCAAAAATGACCACATCAACATC, and a 3′ primer with a *Sac*I cloning site, GAGCTCGAGCTCGAGCTCGAGCTCCTAGAATTGATTGAAAAGTGGG. The PCR product and pOS12, the *col-10* promoter construct (S. Orita and M. Han, unpublished), were digested with *Nhe*I and *Sac*I and ligated.

RESULTS

sem-4 Promotes Vulval Cell Induction

We screened 13,000 EMS-mutagenized haploid genomes for egg-laying-defective mutants associated with abnormal vulval morphology and identified three noncomplementing alleles, *ku200, ku206,* and *ku210,* with defects in the vulval lineages. Mapping data placed *ku200* close to *sem-4* on chromosome I (data not shown, see Materials and Methods). Genetic mapping, cosmid rescue experiments, and complementation tests indicated that *ku200, ku206,* and *ku210* are additional alleles of *sem-4* (Materials and Methods and data not shown). Although a vulval lineage defect had not been previously reported as a *sem-4* mutant phenotype, we observed that *ku200* mutants had no sex myoblasts, similar to existing *sem-4* alleles, and *sem-4(n1971)* mutants have vulval defects similar to those observed in the new alleles (Table 1).

Characterization of *sem-4* alleles indicates that 42% of *sem-4(ku200)* mutants execute an aberrant P7.p lineage (Table 1 and Fig. 2). The most penetrant defect is adoption of the nonvulval, 3° fate (Table 1 and Fig. 2B). A smaller percentage of the time, P7.p adopts one of three intermediate (2.5°) fates: (1) P7.p executes two divisions without proceeding through the final round of division (Fig. 2D), (2) P7.p executes a normal lineage but its descendants fail to integrate with the rest of the vulva (Fig. 2F), or (3) a single cell at the P7.px stage adopts a 3° fate while its sister is fully induced. *sem-4(ku200)* mutants display similar but less penetrant defects in the lineage of P5.p (Table 1). Lineage specification in P6.p, the 1° lineage, appears generally intact

TABLE 1

P5.p and P7.p Adopt Abnormal Fates in *sem-4* Mutants[a]

			P5.p fates[d]			P7.p fates[d]		
Allele	*n*[b]	% wt[c]	2°	2.5°	3°	2°	2.5°	3°
N2	82	100	100			100		
*n*1971 (null)	138	28[e]	57	27	16	50	15	35
ku200	159	41	72	12	15	58	8	34
ku206	87	39	62	17	21	51	15	34
ku210	158	41	76	12	12	55	8	37
n1378	127	50	82	13	5	62	14	24
n2654	77	100	100			100		

[a] P6.p executes a normal lineage, undergoing three rounds of division, although some cells divide with slightly abnormal planes of division.

[b] Number of animals scored.

[c] Percentage of animals in which all VPCs adopted a wild-type fate.

[d] Percentage of cells with indicated fate. 2° cells execute a wild-type lineage (slight aberrations in the plane of division were tolerated) and all cells integrate to form the vulva. 2.5° fate includes P*n*.p's that undergo only two rounds of division, hybrid lineages in which one P*n*.px adopts a 3° fate and its sister adopts a 2° fate, and lineages in which the correct number of divisions occurs but the cells do not integrate with the rest of the vulva. 3° cells divide once and then fuse to the hypodermis.

[e] There are fewer wild-type *n1971* than *ku200* animals even though the total percentage of abnormal P5.p or P7.p cells does not differ greatly because P5.p and P7.p adopt abnormal fates at the same time more often in *ku200* than in *n1971* animals. In contrast, in *n1971* animals, the fates of P5.p and P7.p are more independent of each other.

in *sem-4* mutants. Although we have observed slight defects in the choice of division plane during the terminal division of both the 1° and the 2° lineages (data not shown), P6.p properly executes three rounds of division, and P6.p progeny adopt normal morphologies. In a low percentage (2%, $n = 138$) of *sem-4(n1971 null)* mutant animals, P4.p or P8.p was observed to abnormally adopt a vulval fate (data not shown). *ku206, ku210,* the null allele *n1971,* and the mesodermal-affected allele *n1378* cause a range and severity of phenotypes similar to those of *ku200* (Table 1). However, these phenotypes are not shared with the neuronal-affected allele *n2654* (Table 1).

ku200, ku206, and ku210 Are Strong Loss-of-Function Mutations of sem-4

sem-4 encodes a putative transcription factor with seven zinc fingers (Fig. 3 and Basson and Horvitz, 1996). The primarily mesodermal-affected allele *n1378,* which has a phenotype similar to that of *ku200, ku206,* or *ku210* (Table 1), results in a truncation of the last three zinc fingers of SEM-4 (Basson and Horvitz, 1996). Sequencing of genomic DNA from the *ku200, ku206,* and *ku210* mutants revealed that all three alleles encode predicted proteins with deletions of the last four zinc fingers (Fig. 3). These data further strengthen the hypothesis of Basson and Horvitz (1996) that at least one of the last three zinc fingers is necessary for fate determination of sex myoblasts. Furthermore, we have demonstrated that at least one of the last three zinc fingers plays an important role in vulval cell-fate specification. Consistent with this hypothesis, the lesion in the neuronal-affected allele, *n2654,* which results in a protein with a missense mutation in a conserved residue of the second zinc finger and is not predicted to affect the C-terminal zinc fingers (Basson and Horvitz, 1996), has no effect on vulval cell-fate specification, and the null allele *n1971,* which is predicted to encode a protein truncated for all seven zinc fingers (Fig. 3 and Basson and Horvitz, 1996), does cause defects in vulval cell-fate specification.

Because *ku200, ku206,* and *ku210* encode proteins lacking a specific set of zinc fingers, they are unlikely to bind to the targets of these zinc fingers. Thus, these alleles likely result in a strong loss-of-function phenotype with regard to interaction with or expression of those targets. The severity of the vulval lineage defects in *ku200* compared to that of

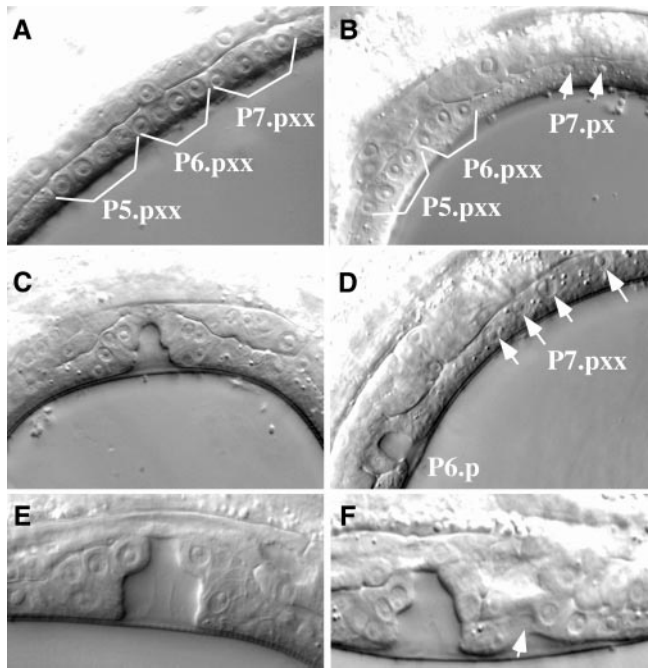

FIG. 2. *sem-4* mutants have reduced vulval induction. DIC photomicrographs of (A, C, E) N2 and (B, D, F) *sem-4(ku200)* hermaphrodites oriented with anterior to the left. (A, B) L3, P*n*.pxx stage. P7.p has adopted a 3° fate (arrows) in *sem-4.* (C, D) Young L4, P*n*.pxxx stage. P7.p descendants have ceased further development after second division (arrows) in *sem-4.* P6.p adopted a proper 1° fate while P5.p is 3° (not shown). (E, F) Mid-L4 stage. P7.p is properly induced but fails to integrate with the other vulval cells (arrow) in *sem-4.*

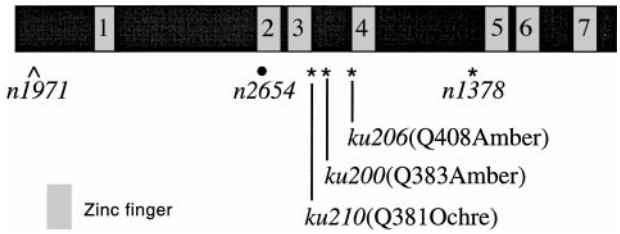

FIG. 3. *ku200*, *ku206*, and *ku210*, which disrupt *sem-4* function in the vulva and mesoderm, specifically disrupt the last four zinc fingers of SEM-4. (^) The null allele, *n1971*, has a lesion in the splice donor consensus of the second exon. Failure to splice introduces an early stop codon that severely truncates the protein (Basson and Horvitz, 1996). Filled dot indicates the tissue-specific lesion, *n2654*, that disrupts the fate specification of several neurons and encodes a missense mutation, H323Y, predicted to abolish function of the second zinc finger (Basson and Horvitz, 1996). Stars indicate tissue-specific lesions which disrupt fate determination of vulval, sex-myoblast, and coelomocyte lineages. Each of these alleles introduces a premature stop codon at the indicated position.

the null (Table 1) is consistent with the hypothesis that *ku200* practically abolishes *sem-4* function during vulval induction. In contrast, only 2% ($n = 100$) of *ku200* animals are constipated compared to 97% (Basson and Horvitz, 1996) of the *sem-4(n1971 null)* animals, indicating that *sem-4* activity in the neuronal lineages, most likely mediated through at least the second zinc finger, is relatively normal in *ku200* mutants. Molecular and phenotypic characterization of *ku200* suggests that the role of *sem-4* is to promote the adoption of vulval fates. Because *ku200*, *ku206*, and *ku210* are markedly similar, both genetically and molecularly, and since each appears to be a strong loss-of-function allele with regard to vulval development, we have chosen to use *ku200* as a representative allele for further analysis.

sem-4 Is Expressed in the Vulval Lineages

We used a *sem-4*::*gfp* translational fusion (kindly provided by M. Koelle) to examine the expression of *sem-4* in the vulva during induction. Consistent with the multiple phenotypes associated with the null mutant, *sem-4*::*gfp* is expressed in a variety of cells, including the HSNs; hyp 8, 9, and 10; B; F; rect D; DVC; some ventral cord neurons; cells in the head; and cells in the preanal ganglion (data not shown; M. Koelle, personal communication; and Basson and Horvitz, 1996). Furthermore, we observed *sem-4*::*gfp* expression in the VPCs, consistent with its role in induction of vulval fates (Fig. 4). Expression in the VPCs is first observable during L2 and persists in the vulval lineages until the vulval divisions are complete (Fig. 4). Expression is

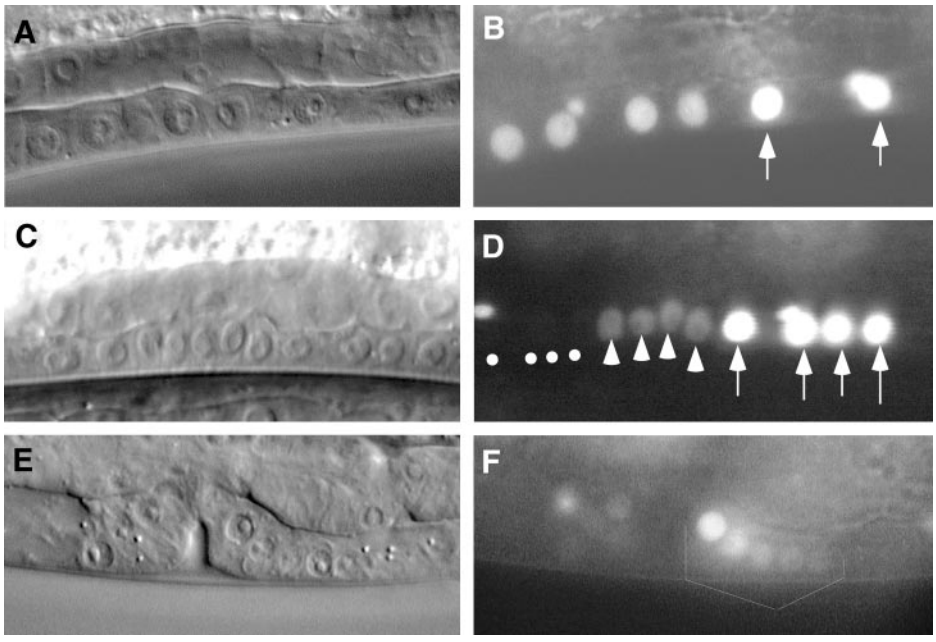

FIG. 4. *sem-4*::*gfp* is expressed in the developing vulva, with stronger expression in P7.p. All images are oriented with anterior to the left and dorsal at the top. (A, C, E) DIC photomicrographs and (B, D, F) corresponding fluorescence images of *unc-119; kuEx113[sem-4*::*gfp unc-119(+)]* hermaphrodites. (A, B) P*n*.px stage. P7.px cells (arrows) have stronger expression than P6.px or P5.px. (C, D) P*n*.pxx stage. Expression is strongest in the P7.p lineage (arrows), weak in the P6.p lineage (arrowheads), and seemingly absent in the P5.p lineage (dots). (E, F) P*n*.pxxx stage (vulval divisions are complete). Expression in P7.p descendants is strongest (brackets).

TABLE 2

sem-4 Has a Strong Genetic Interaction with *lin-39*

Genotype	*n*[a]	P5.p fates[b]			P7.p fates[b]		
		2°	2.5°	3°	2°	2.5°	3°
N2	82	100			100		
sem-4(ku200) I	147	73	12	15	59	8	33
let-60ras(n1046) IV	Many	100			100		
sem-4(ku200) I; *let-60(n1046)* IV	61	89[c]			61[c]	7	7
lin-45raf(ku112) dpy-20(e1282) IV	Many	100			100		
sem-4(ku200) I; *lin-45(ku112) dpy-20(e1282)* IV	27	55	7	37	41	4	55
lin-39(n709ts) III 15°	73	82	6	12	79	6	5
sem-4(ku200) I; *lin-39(n709ts)* III 15°	35	29	17	54	17	3	80

[a] Number of animals scored.
[b] See footnote *d* in Table 1.
[c] Remaining animals (to equal 100%) were induced but had 1° or ambiguous lineages.

undetectable again by the mid-L4 stage, before vulval morphogenesis has terminated (data not shown). Expression of *sem-4::gfp* in vulval cells is often strongest in the descendants of P7.p (Fig. 4), consistent with the higher penetrance of *sem-4* mutant phenotypes in P7.p.

sem-4 Interactions with Vulval-Induction Mutants

Vulval cell differentiation requires signaling through a conserved Ras/Raf/MAPK pathway (reviewed in Kornfield, 1997; Sternberg and Han, 1998). In wild-type animals, the EGF-like ligand LIN-3 signals from the anchor cell to P5.p, P6.p, and P7.p, activating the Ras/Raf/MAPK cascade and leading to the adoption of vulval fates. Lateral signaling involving *lin-12* Notch is required for 2° cell fate determination and in conjunction with Ras-mediated signal transduction is used to pattern P5.p, P6.p, and P7.p to adopt 2°, 1°, and 2° fates, respectively (reviewed in Greenwald, 1997). Mutations that lead to overactive signaling through LET-60 Ras cause additional VPCs (P3.p, P4.p, or P8.p) to adopt vulval fates resulting in a Multivulva phenotype. In contrast, mutations that eliminate signaling lead to a Vulvaless phenotype in which none of the VPCs adopt vulval fates.

To investigate if *sem-4* acts in the Ras signaling pathway, we addressed whether *sem-4* mutations could suppress the Multivulva phenotype of a constitutively active allele of *let-60 ras, n1046.* The *sem-4(ku200); let-60(n1046)* double mutant was Multivulva (data not shown), indicating that *sem-4* cannot suppress LET-60 Ras-mediated signaling and is not acting directly downstream of LET-60 Ras. However, upon scoring vulval induction in the double mutants, we observed an intermediate level of P7.p induction, with fewer P7.p cells adopting the wild-type 2° fate associated with *let-60(n1046)* or the severe 3° fate phenotype associated with *ku200* (Table 2), indicating that excessive LET-60 Ras signaling can partially but not completely compensate for loss of *sem-4* activity. These data suggest that *sem-4* is not acting directly in the Ras/Raf/MAPK signaling cascade but rather is acting in parallel to Ras to affect vulval cell fate.

Constitutive activation of Ras signaling using *let-60 ras(n1046)* causes P8.p to be induced approximately 33% ($n = 27$) of the time. Eliminating *sem-4* activity appears to have minimal effect on P8.p in an otherwise wild-type background. *sem-4(ku200)* mutants have normal induction of P8.p and only 2% of *sem-4(n1971 null)* animals have aberrant induction of P8.p. However, the rate of induction for P8.p in *sem-4(ku200); let-60(n1046)* double mutants doubled to 67% ($n = 61$), suggesting that *sem-4* may play a role in P8.p opposite to the role it plays in P7.p.

We further examined any potential interaction between Ras/Raf/MAPK signaling and *sem-4* by characterizing a double mutant with a weak loss-of-function mutation in *lin-45 raf, ku112.* The *lin-45(ku112)* mutation results in no induction phenotype alone (Table 2 and Sundaram and Han, 1995), but will interact with other mutations that weakly reduce Ras-mediated signaling to produce a Vulvaless phenotype (e.g., Sundaram and Han, 1995). Although we observed some synergy between *lin-45(ku112)* and *sem-4(ku200)* (Table 2), this genetic interaction was weak compared to the strong interactions possible with other components important for optimal signaling (e.g., KSR-1; Sundaram and Han, 1995) and indicates, again, that *sem-4* probably does not act directly in this pathway, but more likely acts in a parallel pathway, to promote vulval induction.

lin-39 has been known to play a role in preventing fusion of the VPCs to hyp7 during the L1 stage, allowing P3.p–P8.p to remain competent to adopt vulval fates in L3 (Clark *et al.,* 1993; Wang *et al.,* 1993). However, more recently, two groups demonstrated that *lin-39* plays a direct role during the induction phase of vulval development (Clandinin *et al.,* 1997; Maloof and Kenyon, 1998). Reminiscent of the phenotype associated with *sem-4(ku200),* when *lin-39* ac-

tivity is reduced during L3, VPCs are resistant to induction, leading to adoption of abnormal hybrid and 3° fates, particularly in P5.p and P7.p (Clandinin *et al.*, 1997; Maloof and Kenyon, 1998). Thus, we tested for genetic interaction between *lin-39* and *sem-4(ku200).* We used a weak loss-of-function, temperature-sensitive mutation in *lin-39*, *n709*, in order to ensure that enough LIN-39 was present to prevent fusion during L1 and allow us to assay vulval cell induction during L3. At the permissive temperature, a significant number of *lin-39(n709)* mutants become VPCs when fusion is avoided during L1. When P5.p and P7.p join the vulval equivalence group in *lin-39(n709ts)* mutants, they are induced with a very high success rate (Table 2). However, when P5.p and P7.p join the vulval equivalence group in *sem-4(ku200); lin-39(n709)* double mutants, they are severely compromised for induction and fully induced with a poor success rate (2.8- to 4.6-fold less effectively, respectively) (Table 2). The dramatic synergy between *sem-4* and *lin-39* suggests that the functions of these gene products are closely related and are involved in determining the fates of vulval cells.

In *sem-4* mutants we do not observe the abnormal F fates that are common in *lin-39* null mutants, suggesting that although *sem-4* plays a role with *lin-39* during vulval induction, a *sem-4- lin-39* interaction is not vital in preventing fusion during L1. However, *sem-4* may contribute slightly to *lin-39* activity during this early stage since the *sem-4(ku200); lin-39(n709)* double mutant did display an increase in abnormal F fates. Only 9–11% (n = 90) of the P5.p or P7.p cells in the *lin-39(n709)* mutant adopted the an F fate at 15°C, leaving approximately 90% of the cells to become VPCs which could be scored in this experiment. In contrast, 40–46% (n = 92) of the P5.p or P7.p cells in the double mutant adopted the F fate, suggesting that when *lin-39* activity is compromised, *sem-4* can contribute to prevention of fusion.

sem-4 Function Is Required for Proper Expression of lin-39 Hox

sem-4 and *lin-39* mutants exhibit similar vulval phenotypes and a strong genetic interaction. Thus, we investigated whether the transcription factor SEM-4 affects expression of *lin-39* by comparing *lin-39::lacZ* expression in wild-type and *sem-4(ku200)* genetic backgrounds (Wang *et al.*, 1993). No significant differences were seen in staining patterns when examining early larval stages (data not shown). However, in late L2 and older animals, 90% (n = 102) of control animals displayed X-gal staining in the vulval lineages compared to only 41% (n = 169) of animals with a *sem-4(ku200)* genetic background. Additionally, staining in the *sem-4(ku200)* background was often significantly less intense than in controls (Figs. 5A and 5B). Although *lacZ* staining in the vulva was severely reduced, *sem-4(ku200)* animals were not resistant to the staining procedure since *lin-39::lacZ* expression was still evident in the eggs and L1 larvae (data not shown). As a more sensitive assay for *lin-39::lacZ* expression, we also performed immunofluorescence with anti-β-galactosidase antibodies on animals carrying the *lin-39::lacZ* reporter fusion (Figs. 5C–5F). One hundred percent (n = 29) of control animals exhibited fluorescent signal in the vulva. However, only 66% (n = 30) of animals with the *sem-4(ku200)* background had detectable *lin-39::lacZ* expression, and, again, many mutant animals exhibited a less intense signal (Figs. 5D and 5F).

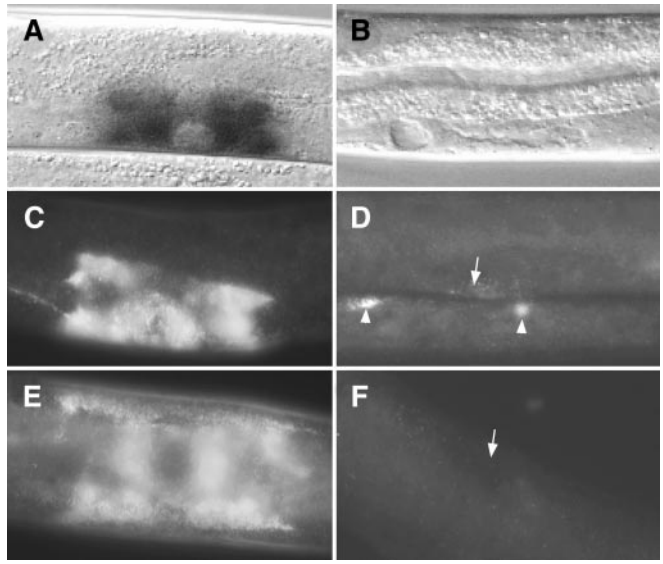

FIG. 5. *lin-39* expression is reduced in *sem-4* mutants. (A, C, E) *muIs6[lin-39::lacZ pRF4]* hermaphrodites. (B, D, F) *sem-4(ku200); muIs6* hermaphrodites. (A, B) Lateral view of mid-L4 animals stained with X-gal. β-Gal activity is severely reduced in the *sem-4* mutant. (C, D, E, F) Immunofluorescence with anti-lacZ antibodies. (C, D) Lateral view of late L3 to early L4 hermaphrodites. Arrow indicates animal with weak expression in the vulva. Arrowheads indicate fluorescence in the VPCs of an L1 hermaphrodite, indicating that LIN-39 is present early. (E, F) Ventral views of mid-L4 vulva showing that β-galactosidase is undetectable in the mutant. Arrow indicates the position of the vulva.

Finally, as further confirmation of alterations in expression, we used an anti-LIN-39 polyclonal antibody to examine expression of LIN-39 directly (Maloof and Kenyon, 1998). Results were consistent with the analysis of the *lin-39::lacZ* reporter construct. Only 9% (n = 34) of wild-type animals failed to display LIN-39 staining or displayed a faint level of staining in vulval cells during induction in L3 or in early L4. In contrast, in 51% (n = 35) of *sem-4(ku200)* animals staining was either faint or absent in all or part of the vulva. The level of decrease in *lin-39::lacZ* or LIN-39 expression in the *sem-4(ku200)* mutant background loosely corresponds with the decrease in percentage of *sem-4(ku200)* hermaphrodites that have wild-type vulval lineages. These data suggest that *sem-4* acts positively to maintain *lin-39* expression and that *sem-4*

TABLE 3

Overexpression of *lin-39* from the *col-10* Promoter Rescues the *sem-4* Vulval-Induction Defects [% *sem-4(ku200)* Animals with Wild-Type VPC Induction][a]

Line	Without transgene	With *col-10::lin-39* transgene	*col-10::lin-39* concentration	Rescue[b]	
A	27 (n = 136)	64 (n = 121)	12 ng/μl	+	z = 5.8
B	32 (n = 96)	43 (n = 86)	12 ng/μl	−	z = 1.5
C	28 (n = 112)	50 (n = 127)	12 ng/μl	+	z = 3.6
D	34 (n = 70)	36 (n = 80)	12 ng/μl	−	z = 0.25
E	27 (n = 123)	45 (n = 103)	36 ng/μl	+	z = 2.7
F	32 (n = 147)	46 (n = 153)	36 ng/μl	+	z = 2.5

Note. n is the number of animals scored.

[a] Animals had normal induction and all vulval cells were integrated into the vulva.

[b] Significant difference demonstrated using z test statistic to test hypotheses regarding binomial population proportions. $z > 2.33$ = significant difference at $\alpha = 0.01$.

may act to promote vulval induction by regulating *lin-39* hox expression.

Ectopic Expression of lin-39 Can Rescue sem-4(ku200)

If up-regulation of *lin-39* expression in response to SEM-4 is necessary for proper induction, then overexpression of *lin-39* in the vulva, independent of SEM-4 activity, would be predicted to rescue the vulval defects of a *sem-4* mutant. This hypothesis was tested by using *col-10* and heat shock promoters to ectopically express LIN-39 in vulval tissue. *col-10* encodes a collagen gene that is expressed in epidermal cells (including the specialized vulval epidermal lineage), beginning in embryogenesis and extending through the period of vulval induction (Cox and Hirsh, 1985), whereas the heat-shock promoter causes ubiquitous expression upon heat shock.

sem-4(ku200) animals were injected with *col-10::lin-39* at two concentrations in conjunction with a GFP-expressing construct to monitor maintenance of the resulting extrachromosomal array. When comparing animals that maintained the array with those that lost the array, we observed a significant difference in percentage of fully induced vulvae (Table 3). Although this rescuing activity was not robust, rescue activity was observed in multiple lines at both concentrations of *col-10::lin-39*, indicating that ectopic expression of LIN-39 can partially compensate for the *sem-4(ku200)* defect. We also examined the ability of ectopic expression of *lin-39* to rescue *sem-4(ku200)* when expressed from the stronger heat-shock promoter. An integrated array, *muIs26*, carrying the *hs::lin-39* fusion (gift from Maloof and Kenyon) was crossed into a *sem-4(ku200)* background. Even in the absence of heat shock, some rescue of the *sem-4(ku200)* induction defect was evident (Table 4). However, when a high, consistent level of LIN-39 was provided under heat-shock conditions, rescue, as assayed by percentage of wild-type vulvae, was markedly increased to the level of 78% wild type (Table 4). LIN-39 overexpression in this experiment began during L2 and was maintained throughout the remainder of vulval development by alternating the animals between 20°C and a 2-h heat pulse at 30°C every 2 h until the L4 stage, at which point vulval induction was scored.

Since neither overexpression protocol provided complete rescue of *sem-4(ku200)*, it remains possible that *sem-4* acts on an additional factor or factors to provide the remaining contribution to the vulval induction pathway. Alternatively, the strong heat-shock promoter may have failed to provide complete rescue because the heat-shock regimen caused slow growth in the animals carrying the extrachromosomal array, and these unhealthy animals may be unable to execute vulval induction normally. Despite this, rescue was quite robust. The rescue of *sem-4(ku200)* by overexpression of *lin-39* combined with the reduction in *lin-39* expression observed in *sem-4* mutants supports the hypothesis that *sem-4* regulates vulval induction by positively regulating *lin-39* activity in the vulval lineages.

TABLE 4

Overexpression of *lin-39* from a Heat-Shock Promoter Rescues *sem-4* Vulva-Induction Defects

Strain	Heat shock	n[a]	% wt animals[b]
sem-4(ku200)	−	159	41
sem-4(ku200)	+	54	39
sem-4(ku200) I; *dpy-20(e1282)* IV; *muIs23*	−	32	53
sem-4(ku200) I; *dpy-20(e1282)* IV; *muIs23*	+	46	78[c]

[a] Number of animals scored.

[b] Percentage of animals with wild-type induction and integration of all vulval cells into the vulva.

[c] Significantly different from *sem-4* subjected to heat shock without the array. z = 3.9. See footnote *b* to Table 3.

Expression of sem-4 Appears to Be Regulated by LIN-12

Interestingly, loss of *sem-4* activity has a greater effect on 2° than on 1° lineages and has its strongest effect specifically in P7.p. Redundant mechanisms for maintaining *lin-39* activity in P6.p may exist such that the 1° lineage is not as sensitive to *sem-4* activity as the 2° lineages. This is consistent with the demonstration that although *lin-39* is up-regulated in all VPCs at the time of induction, up-regulation is most evident in P6.p (Maloof and Kenyon, 1998). Alternatively (or in conjunction with this first mechanism), *sem-4* might be specifically required for the transduction of the LIN-12/Notch lateral signal, thereby affecting the 2° cells to a greater degree. If SEM-4 is involved in transcription of downstream targets of LIN-12, loss of *sem-4* could affect induction of P5.p and P7.p. A potential role for *sem-4* in lateral signaling is supported by an observation of the *sem-4(ku200); let-60ras(gf)* double mutant. Although extra VPCs are induced in a *let-60ras(gf)* background, an alternating pattern of 1° and 2° lineages is always observed such that two primary lineages are never adjacent. This alternating pattern requires LIN-12 signaling activity (Greenwald *et al.*, 1983; Sternberg, 1988). In contrast, in the *sem-4(ku200); let-60ras(gf)* double mutant, adjacent 1° fates are occasionally observed (data not shown), suggesting that loss of *sem-4* activity can interfere with lateral signaling. We further investigated a potential role of *sem-4* in LIN-12/Notch signaling by examining the expression pattern of *sem-4*::*gfp* in *lin-12* mutant backgrounds.

We placed the integrated array encoding *sem-4*::*gfp* in a *lin-12* loss-of-function as well as a *lin-12* gain-of-function mutant background. In the *lin-12(lf)* background we monitored expression of the array in the 1° lineage of P6.p since all VPCs adopt a 1° fate in a *lin-12(lf)* mutant. In a wild-type background, 12% of the animals express weak levels and 28% of the animals express strong levels of *sem-4*::*gfp* in P6.p ($n = 25$). In contrast, we could not detect expression in P6.p in a *lin-12(lf)* background ($n = 13$), consistent with a role for LIN-12 in positively regulating *sem-4* expression. In the *lin-12(gf)* mutant we monitored expression in the 2° lineage of P7.p since all cells adopt a 2° fate in the gain-of-function background. However, expression was not obviously enhanced above the already strong levels seen in the wild-type animals. In a wild-type background, 19% of the animals express weak levels and 76% of the animals express strong levels of *sem-4*::*gfp* in P7.p ($n = 21$). We detected expression in P7.p in a *lin-12(gf)* background in all animals ($n = 12$), although expression was classified as strong in 50% of the animals.

mab-5 May Contribute to the Increased Sensitivity of P7.p to sem-4(ku200)

One intriguing aspect of the *sem-4* mutant phenotype is the stronger penetrance in P7.p compared to P5.p. Single P*n*.p isolations show that P7.p is not as competent as P5.p to activate LET-60 Ras signaling due to the action of the hox gene *mab-5* (Clandinin *et al.*, 1997), which is expressed in P7.p–P9.p (Salser *et al.*, 1993). To test whether *mab-5* contributes to the higher penetrance of *sem-4(ku200)* in P7.p, we constructed double mutants between loss-of-function *mab-5(e1239)* and *sem-4(ku200)*. If *mab-5* activity accounts for some of the difficulty in inducing P7.p in a *sem-4* mutant relative to P5.p, which does not express *mab-5*, then we would expect that in the double mutant, P7.p would be easier to induce. Indeed, this was the case, although the effect was not very strong. The same percentage of P7.p cells adopted wild-type 2° fates in the double mutants as in *sem-4(ku200)*. However, only 6% ($n = 42$) of P7.p cells were completely uninduced (adopted a 3° fate) in the double mutant compared to 34% ($n = 159$) in *sem-4(ku200)*. One caveat to this experiment is that P5.p induction was worsened in the double mutant. *mab-5* expression cannot be detected in P5.p, but *mab-5* is known to actually promote vulval fate in P5.p, consistent with this result (Clandinin *et al.*, 1997).

DISCUSSION

sem-4 Plays a Role in Vulval Cell Induction

We have identified three additional alleles of *sem-4* and characterized a previously unreported, loss-of-function phenotype. These alleles fall into the mesodermal-affected class identified by Basson and Horvitz (1996). We have demonstrated that alleles of this class, as well as the null allele, are associated with vulval lineage defects. Specifically, loss of *sem-4* activity in the vulval lineages results in a high incidence of abnormal adoption of 3° and hybrid fates in place of 2° fates and affects P7.p more strongly than P5.p. Less frequently, a normal 2° lineage may be specified but the mutant vulval cells invaginate and undergo morphogenesis independently, indicating a failure to properly coordinate development with other vulval cells.

sem-4-associated vulval phenotypes resemble those of animals that have lost LIN-39 activity after the L1 fusion event (Clandinin *et al.*, 1997; Maloof and Kenyon, 1998). We have established that in *sem-4* mutants *lin-39* expression is decreased and ectopic expression of *lin-39* can provide partial rescue, suggesting that *sem-4* acts upstream of *lin-39* in vulval induction and partially exerts its effects by regulating *lin-39*. However, it is possible that *sem-4* plays a role in vulval induction in addition to those mediated by *lin-39* since *hs*::*lin-39* expression at the highest levels does not completely rescue the *sem-4* vulval defects.

Positive Regulation of lin-39 by sem-4 Is a Relatively Late Event

In *sem-4* mutants we do not observe P*n*.p cells that adopt the abnormal F fates that are associated with strong loss-of-function mutations in *lin-39* or with other mutants, such as *bar-1*, known to regulate *lin-39* during early larval stages (Eisenmann *et al.*, 1998). The reduction of *lin-39* expression

observed in a *sem-4* mutant background is a relatively late event. Expression of *lin-39* is normal during the early larval stages (see Fig. 5D) and is reduced only during induction and execution of vulval fates, indicating that *sem-4* is not required for establishing *lin-39* expression, but more likely acts to maintain appropriate levels of *lin-39* in specific vulval cells during induction. Even during L3, *lin-39* activity is not eliminated with 100% efficiency in *sem-4* mutants. Approximately 50% of *sem-4* mutants still express *lin-39*, suggesting that factors in addition to SEM-4 act to positively regulate *lin-39* expression.

lin-39 is absolutely required for induction (Maloof and Kenyon, 1998). Furthermore, *lin-39* is a factor that provides specificity to Ras signaling in the VPCs, such that vulval fates are the outcome of signaling (Maloof and Kenyon, 1998). Because *lin-39* plays such a pivotal role, establishment and maintenance of *lin-39* expression are critically important and appear to be controlled by many factors. In addition to the regulation of *lin-39* by *sem-4* as described here and by Ras-mediated signal transduction and BAR-1, as previously mentioned, regulation by EGL-27 and LIN-39 itself has been established as critically important for maintaining appropriate levels of *lin-39*. *egl-27* encodes a *C. elegans* homolog of MTA1, a component of a nucleosome remodeling and histone deacetylation complex (Ch'ng and Kenyon, 1999). *egl-27* functions to regulate both the expression of *lin-39* and how *lin-39* and *mab-5* functionally interact. Additionally, LIN-39 protein itself is required for the observed up-regulation of *lin-39* expression in response to Ras-mediated signaling (Maloof and Kenyon, 1998). It is still vague at this point how each of these factors contributes to establishment or critical maintenance of *lin-39* expression in the vulva and how interplay between these various factors is coordinated or regulated. Although *lin-39* appears to act downstream of *sem-4* during vulval induction, whether SEM-4 directly or indirectly affects *lin-39* expression requires further investigation.

sem-4 May Also Affect Expression or Activity of mab-5

P7.p is most sensitive to loss of *sem-4* activity (Table 1), but P5.p is most likely to have a defective lineage when *lin-39* activity is compromised (Clandinin *et al.*, 1997; Maloof and Kenyon, 1998), suggesting that *sem-4* function may be mediated or affected by other factors in addition to *lin-39*. Furthermore, although the stronger expression of *sem-4*::*gfp* in P7.p may explain the more strict reliance of P7.p on *sem-4* compared to P5.p, additional factors may be important for this phenomenon as well. We originally hypothesized that the higher penetrance of the *sem-4* phenotype in P7.p might be due to the action of *mab-5*, which has been shown to have slightly inhibitory effects on the induction of P7.p (Clandinin *et al.*, 1997). The increased level of SEM-4::GFP in P7.p may reflect the need to overcome inhibitory effects of *mab-5*. While, as expected, induction of P7.p is slightly improved in the *sem-4(ku200); mab-5(e1239lf)* double mutant compared to the *sem-4(ku200)* mutant, the integration defect causing P7.p to fail to join the other vulva cells even when induced is exacerbated slightly (data not shown). This suggests that *sem-4* and *mab-5* may act together to affect induction as well as morphogenesis.

Regulation of sem-4

Since *sem-4* mutants do not share the F fate phenotype observed in *bar-1* mutants, it seems unlikely that *sem-4* is a downstream component of a wnt signaling pathway acting to regulate *lin-39* activity during L1 or L2. It is more likely that *sem-4* acts to maintain *lin-39* expression only at the time of induction. Our evidence suggests that this regulation may in part be in response to LIN-12-mediated signaling. Additionally, *sem-4* could coordinate input from the Ras-mediated signaling pathway or yet another pathway controlling the regulation of *lin-39* during vulval induction and morphogenesis. We examined the pattern of *sem-4:gfp* expression in mutant backgrounds in which Ras-signaling was compromised or constitutively active without noticing any obvious effects on expression (data not shown). However, it remains possible that SEM-4 activity may be altered by Ras-mediated signal transduction even if SEM-4 expression is not. In summary, identification of *sem-4* as a regulator of *lin-39* expression adds to our understanding of how *lin-39* expression is maintained in the vulva but brings up many more interesting questions for future investigation about how interplay between regulators of *lin-39* coordinates all incoming signals, resulting in appropriate spatial and temporal levels of *lin-39* activity leading to vulval induction.

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